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The proposal will test the hypothesis that bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. Further, that cells in breast cancer bone metastases are specialized populations of cancer cells, endowed with properties that promote their growth in bone. The presence of breast cancer cells can disrupt the normal balance of bone turnover and promote osteoclast activity. Understanding the biology of breast cancer bone metastases and the contribution of cancer cell-derived factors, such as platelet derived growth factor (PDGF), will lead to new approaches for control or prevention of this significant clinical problem. Expression analyses will be performed using cDNA arrays, testing samples from breast cancer cell lines growing in different conditions – *in vitro* and *in vivo* (direct injection into bone or mammary fatpad, and/or metastases from different organs in mice). The arrays to be used will identify cytokines and receptors, and genes involved in specific pathways (cell cycle regulation, cell death, metastasis and invasion, signal transduction, angiogenesis). One of the factors known to promote bone resorption is PDGF, and the consequences of the release of PDGF by metastatic breast cancer cells will be determined in *in vitro* experiments with immortalized osteoblasts.

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Tumor-host interactions in breast cancer bone metastasis

INTRODUCTION:

Breast cancer is the most common cancer (after non-melanoma skin cancer) of women in the United States of America. Twenty percent of women with early stage, node-negative breast cancer may subsequently develop metastatic disease, while as many as 90% of women with locally advanced, or with extensive lymph node involvement will develop metastases (1). In addition to the axillary lymph nodes, other sites where breast cancer metastases are found include the liver, lungs and brain. However, the most common site of breast cancer metastasis is the bone (2-4). Breast cancer metastases in bone predominantly present as osteolytic lesions. Such lesions can have serious complications, including hypercalcemia, pain, pathologic fractures and central nervous compromise (spinal cord or nerve root compression)(5). Bone metastases are the most common cause of pain for cancer patients, resulting from either mechanical or chemical stimulation of pain receptors in the periosteum or endosteum. Pressure effects, microfractures and cytokine release also contribute to the pain (6). Patients with bone as the first site of relapse of breast cancer can have a significantly longer survival than patients with liver as the first site (20 months vs. 3 months median survival after relapse) (5). However, the prolonged course of a disease with such complications as bone pain and pathological fractures severely reduces a patient's quality of life, and can make heavy demands on health care resources. There is increasing support for the idea that certain properties of breast cancer cells contribute to the high incidence of bone metastases of this disease. One observation is the correlation between expression of parathyroid hormone related protein (PTHrP) and breast cancer bone metastasis (7). PTHrP has been shown to be upregulated in breast cancer cells exposed to TGF- β 1(8). This may serve to enhance PTHrP expression locally, since the bone matrix is a rich source of a variety of growth factors, including insulin-like growth factors I and II (IGF-I, IGF-II) and TGF- β (9; 10).

In healthy adult bone there is a continuous process of turnover with a balance maintained between resorption and new bone formation. In post-menopausal women or in conditions of estrogen depletion the balance of normal bone turnover can become uncoupled. Osteoblast function is reduced while osteoclast function is maintained, leading to net bone loss. Multiple cytokines and hormones are involved in osteoblast stimulation of osteoclastogenesis. Many of these, for example PTHrP, IL-6, IL-11 and M-CSF, are expressed by tumor cells (11). Another key factor is the TNF-like cytokine osteoprotegerin ligand (OPGL), also known as RANKL (receptor activator of NF- κ B ligand) or ODF (osteoclast differentiation factor). The receptor RANK is expressed by osteoclasts. The action of OPGL is antagonized by a soluble "decoy" form of the receptor, osteoprotegerin (OPG), which is also expressed by osteoblasts (12) suggesting a finely tuned system for the local control of osteoclast activation. Exposure of osteoblasts to cytokines and other factors (including PTHrP) that promote bone resorption can stimulate expression of OPGL and reduce levels of OPG (13; 14). Bone resorption leads to the local release of matrix-bound factors and cytokines that normally stimulate osteoblasts to form new bone (9), yet could also promote the survival and growth of breast cancer cells. As noted above, TGF- β , which is abundant in bone matrix, can increase PTHrP production by metastatic breast cancer cells. In a preliminary study we found that TGF- β treatment of breast cancer cells increased the release of PDGF, another factor which can stimulate bone resorption. Others have shown that the media from cultures of resorbing bone are chemotactic and growth stimulatory for rat and human breast cancer cells (15; 16). Breast cancer bone metastases are commonly found

in sites of active bone remodeling, predominantly in trabecular bone, suggesting that the growth and survival of metastatic cells are promoted in areas of remodeling one.

The hypothesis tested in this work is that bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. Further, that cells in breast cancer bone metastases are specialized populations of cancer cells, endowed with properties that promote their growth in bone. The presence of breast cancer cells can disrupt the normal balance of bone turnover and promote osteoclast activity. Understanding the biology of breast cancer bone metastases and the contribution of cancer cell-derived factors, such as platelet derived growth factor (PDGF), will lead to new approaches for control or prevention of this significant clinical problem.

BODY:

Specific Aim #1: Gene expression comparisons of breast cancer cells growing *in vivo* and *in vitro*

Analyses of gene expression were proposed using two breast cancer cell lines, injected into nude mice to generate tumors, either in the mammary fatpad (as previously described (17)), or into the tibia, which is a model for growth of cancer in the bone (18; 19). The cell lines proposed, BBM1 and SUM149, were determined in preliminary data to be able to grow in the tibia of nude mice, and also in the mammary fatpad. However, in our initial experiments, the cell lines have failed to produce a reliable incidence of bone tumors, such that the recovery of tumor tissues for isolation of RNA for expression analyses has not yet been accomplished with these cell lines. Ongoing experiments are testing different conditions (earlier passage cell cultures, or increased cell inoculum) to achieve the required tumor growth, and to then prepare the material required for genetic analyses.

In the interim, and as an alternative for these two cell lines, the following results have been obtained using two additional human breast cancer cell lines, MDA-MB-231 and MDA-MB-435. Both form lytic tumors following injection into the tibia of nude mice. The temporal sequence of changes in the bone that occur as tumor grow has been determined by killing mice at different times after injection, and preparing histological sections. Two weeks after the injection of 5×10^5 cells, small tumors can be seen in the tibias from mice injected with both cell lines, although no sign of lysis is obvious in radiographs of the limbs (Appendix Fig. 1). After 3 and 4 weeks, the MDA-MB-435 tumor cells cavity of the bone, replacing the normal marrow, yet with little evidence of lytic destruction until later weeks, when the tumors are larger, and begin to grow outside of the bone in surrounding muscles. In contrast the MDA-MB-231 tumors, have a similar time-course of growth, yet show evidence of activation of osteoclasts and bone lysis at earlier stages of tumor growth. Radiography showed the presence of lytic tumors approximately 4 weeks after injection (data not shown). The images shown in Fig. 1 were of histochemistry for tartrasine-resistant acid phosphatase (TRAP), a marker of activated osteoclasts (20). The results suggest that the two breast cancer cell lines differ in the ability to promote activation of the cells principally responsible for bone destruction. Continuing studies using these tissue samples are evaluating expression of different cytokines that promote osteolysis (including IL-6, PTHrP, M-CSF, and RANKL). Expression will be examined in the tumors in the bone, and also in tumors from the mammary fatpad, to test whether the microenvironment in which the tumor cells are growing can influence expression of these factors.

One factor that we hypothesize to be an important player in tumor-host interactions of breast cancer bone metastasis is the platelet-derived growth factor (PDGF) family of growth factors. PDGFs are among the cytokines and growth factors released by breast cancer cells that have the potential for promoting bone resorption (21; 22). The PDGFs form a family of disulfide binding dimeric isoforms; at present there are four known isoforms, A, B, C, and D, and two of these (C and D) require proteolytic activation (23; 24). PDGF A and B can form either homo- or heterodimers, and different cell types differ in expression of the PDGF isoforms. The two specific receptors, PDGF α - and β -receptor are members of the tyrosine kinase receptor superfamily. Ligand binding promotes dimerization of receptor subunits, and triggers tyrosine-specific phosphorylation, initiating a signal transduction cascade and ultimately phenotypic changes (25; 26). PDGFs are potent bone mitogens which stimulate proliferation of osteoblasts, and also increase bone resorption, probably by increasing osteoclast number (21;27). Osteoblasts express receptors for PDGF, and respond to the factors with various phenotypic changes, including upregulation of IL-6 (28). There is less information on the actions of PDGFs directly on osteoclasts, although there is one report that these cells express PDGF receptors (22).

Preliminary data showed that the breast cancer cell lines expressed PDGFs (detected by ELISA), and this has been confirmed by immunohistochemistry (Appendix Fig. 2). This figure shows immunohistochemistry using antibodies detecting PDGF-A, PDGFR α and the phosphorylated form of the receptor (activated PDGFR α), on tumors of the MDA-MB-435 cell line in the mammary fatpad and the tibias of nude mice. While the ligand and receptor are present in the tumors from both sites, there is substantially more activation of the receptor in the bone tumors than in the mammary fatpad tumors. Similar results were found using antibodies against PDGF-B and PDGFR β (not shown). Experiments are planned to use laser scanning cytometry to objectively measure the levels of antibody binding in these specimens, and to test whether the ligand and/or receptor are present in greater abundance in the bone tumors, which might account for the increased receptor activation. TGF- β , a cytokine released from bone matrix by the action of osteoclasts (9) has been reported to increase expression of PDGF in breast cancer cells (29), and we have confirmed this *in vitro* by ELISA measurements of supernatant from MDA-MB-231 and MDA-MB-435 cells treated with TGF- β (Appendix, Fig. 3). Whether TGF- β contributes to increased PDGF expression in bone tumors remains to be established. The results in Fig. 2 suggest differential regulation of PDGF-receptor activation of breast cancer cells growing in different tissue environments. The receptor may be mediating paracrine and autocrine interactions, since we demonstrate the presence of the ligand in the same tumors. Ongoing studies are measuring cell proliferation and death in the tumors, by staining the same tissue sections for proliferating cell nuclear antigen (PCNA) and TUNEL (a marker of apoptotic cells), as used previously (19), as potential indicators of outcome of PDGF-receptor activation in the tumor cells. How the difference in receptor activation relates to the expression of other cytokines involved in promotion of osteoclast activation will be determined by immunohistochemistry using the tissue specimens already available, and then using gene expression arrays from RNA isolated from xenograft tumors.

Specific Aim #2 PDGF-mediated regulation of osteoblast expression of osteolytic cytokines

This aim employs a SV40-large T antigen transformed human fetal osteoblast cell line. These cells, grow actively at 34°C, and grow slowly and differentiate at the permissive

temperature of 39° C (30). Studies of PDGF receptor phosphorylation have been performed in cells at the permissive temperature, i.e. differentiated phenotype. As shown in Fig 4 A (Appendix), the osteoblasts express PDGFR β , which is phosphorylated in response to stimulation by PDGF (in this example using PDGF BB). The tyrosine kinase inhibitor STI571 (Fig 4.B) can inhibit the phosphorylation. Fig 4C shows that the receptor can be stimulated in osteoblasts exposed to conditioned medium collected from breast cancer cells, and that this can be partially inhibited in the presence of 0.5 μ M STI571. The conditioned medium is collected after 48-h culture of the breast cancer cells with serum-free medium. The figure shows the result using conditioned medium from the BBM1 breast cancer cell line. Experiments with conditioned medium from other cell lines (including MDA-MB-231 and MDA-MB-435) are in progress.

The expression of RANKL and OPG by hFOB1.19 cells was measured using quantitative RT-PCR. Total RNA was isolated from hFOB1.19 cells grown in the presence of 1 or 10 ng/ml PDGF BB, and used to determine whether the growth factor altered expression. The initial results show that PDGF stimulation increases the abundance of RANKL in osteoblasts, yet has no effect on the level of OPG expression. (Fig. 5, Appendix). As RANKL is considered a osteoclast-promoting factor, the activity of which can be balanced by the competing decoy receptor OPG, the net effect would be to promote osteoclast activity in the presence of PDGF. Additional cytokines to be measured in further studies include PTHrP, M-CSF and IL-6.

Progress with this aim has been slowed by problems with the culture of the hFOB1.19 cells, which have a long-doubling time (> 48 h) and are very sensitive to culture conditions. For example, if the cultures become over confluent the cells detach and die. Alternative cell lines being considered for use in this part of the study include two human osteosarcoma cell lines (SAOS-2, MG63), both of osteoblastic phenotype, and which express receptors for PDGF (data not shown). Although these are not normal osteoblast cells, they may provide a model with which to examine regulation of cytokines involved in osteoclast activation by PDGFs.

KEY RESEARCH ACCOMPLISHMENTS

- A description of a time-course of the growth of human breast cancer cells in the bone.
- Demonstration of differential activation of PDGF-receptors in breast cancer cells growing in the bone and in the mammary fatpad.
- Demonstration of activation of PDGF receptors of cultured osteoblasts by recombinant PDGF and by culture supernatant from human breast cancer cells, indicating that the cancer cells release biologically active PDGF.
- Demonstration that PDGF can increase expression of RANKL by cultured osteoblasts, but does not alter OPG expression.

REPORTABLE OUTCOME

None to report.

CONCLUSIONS:

This grant is testing the hypothesis that the bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. One result of the studies so

far is that growth in the bone lead to enhanced activity of the PDGF-receptor in breast cancer cells compared with the same cells growing in the mammary fatpad of mice. This result confirms microenvironmental regulation in the breast cancer cells, which will be examined further in the continuing studies of gene expression patterns of breast cancers growing in the different organs (mammary fatpad or bone). PDGFs may have multiple actions in the bone microenvironment, acting on the breast cancer cells and also on osteoblasts, by regulating a key cytokines (for example RANKL) involved in activation of osteoclasts, the cell responsible for bone destruction seen in lytic metastases.

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APPENDIX

FIGURE LEGENDS

Figure 1: Development of tumors following intratibial injection of MDA-MB-231 and MDA-MB-435 breast cancer cells. Mice were killed at intervals following tumor injection, and the lower hind limbs fixed in 10% buffered formalin, then decalcified in 12% EDTA for 8 – 10 days. The tissues were then embedded in paraffin, and 4 μ sections cut and stained for the presence of tartrazine-resistant acid phosphatase positive cells, a marker of activated osteoclasts. The figure shows images from sections of tumors taken at 2, 3 and 6 weeks after injection. The TRAP-positive osteoclasts are visible at the interface of tumor and bone in the earliest samples of MDA-MB-231 tumors, while they are not apparent until 6 weeks after injection in the MDA-MB-435 samples.

Figure 2: Expression of PDGF and PDGFR α in MDA-MB-435 tumors in the mammary fatpad and the bone. MDA-MB-435 cells were injected into the two sites of nude mice, and tissues collected at autopsy 8 weeks later. The tissues were fixed for paraffin embedding (after decalcification as described for Fig. 1), or for frozen sections (fixation in periodate-lysine-paraformaldehyde, decalcified, then embedded in OCT and frozen). Antibodies to PDGF A, PDGFR α (paraffin sections and horseradish peroxidase detection) and the activated form of the receptor (frozen sections, fluorescent detection) were applied to 4 μ sections of the tissue samples. Ligand and receptor were detected in all tumors in both sites, although staining was more intense in the bone tumors. The level of activation of the PDGFR α was markedly more intense in the bone tumors.

Figure 3: PDGF expression in MDA-MB-231 and MDA-MB-435 breast cancer cells in vitro.

Figure 4: Phosphorylation of PDGFR β in hFOB1.19 osteoblasts

A: Cells were serum-starved, then stimulated for 15 min with PDGF BB (0.1 – 25 ng/ml). Cell lysates were prepared, and immunoprecipitation performed using polyclonal antibody to PDGFR β . Precipitated proteins were separated on SDS-PAGE gels, and the presence of tyrosine phosphorylated receptors detected using antibody to phosphotyrosine. A band corresponding to 190kDa was found in samples only from the PDGF stimulated cells.

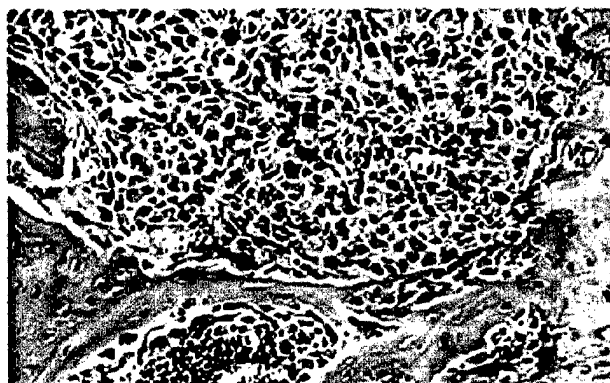
B. Cells were treated with or without the indicated concentrations of the tyrosine kinase inhibitor STI 571 for 30 min prior to stimulation with PDGF, to demonstrate that the inhibitor can prevent activation of the receptor in osteoblasts. Lysates were collected and immunoprecipitation for PDGFR β and blotting for phospho-tyrosine performed.

C. Cells were stimulated with either PDGF or conditioned medium collected from confluent cultures of BBM1 breast cancer cells, to demonstrate the activation of PDGFR β by factors released by cancer cells. Lysates were collected and immunoprecipitation for PDGFR β and blotting for phospho-tyrosine performed. The filter was re-probed for expression of the total levels of receptor.

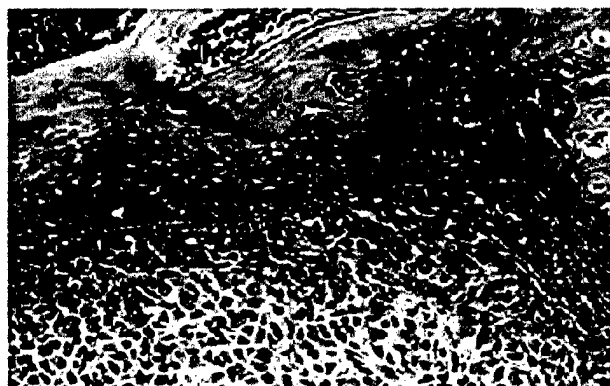
Figure 5: Expression of RANKL and OPG in hFOB1.19 osteoblasts stimulated with PDGF.

MDA-MB-231

Week 2



Week 3



Week 6

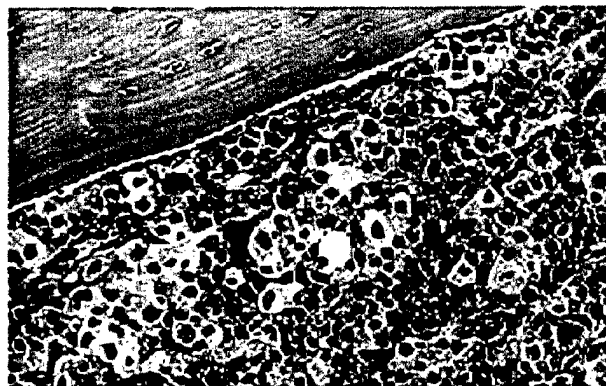


MDA-MB-435

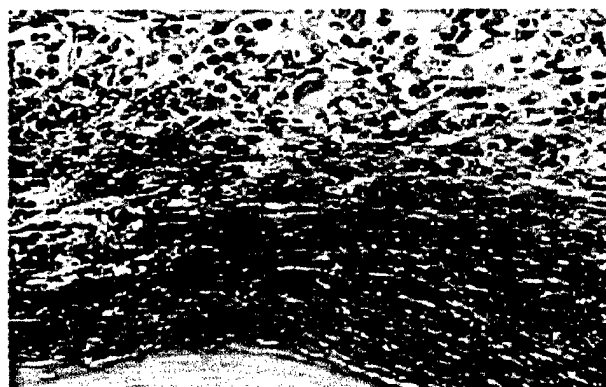
Week 2



Week 3



Week 6



Bright red TRAP (tartrasine-resistant acid phosphatase) -positive activated osteoclasts are more abundant, and appear earlier in the MDA-MB-231 tumor in bone.

Figure 2

MDA-MB-435 breast cancer xenografts

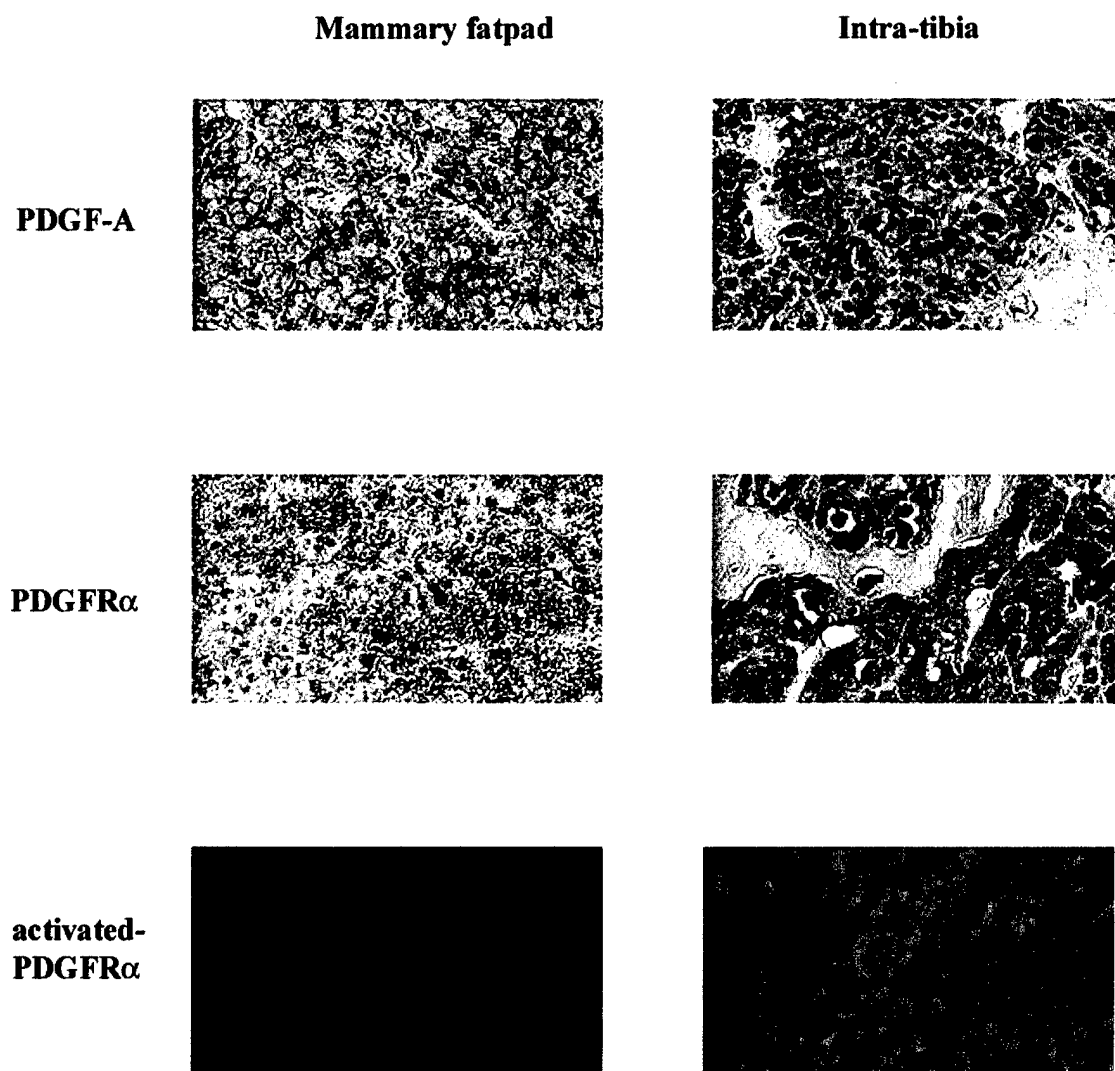
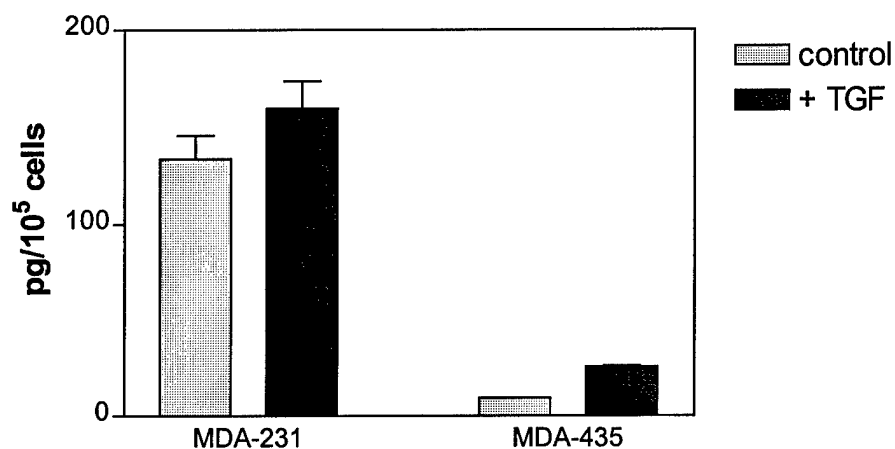


Figure 3:

PDGF AB measured in the supernatant of breast cancer cells cultured with TGF- β



Culture supernatants were collected after 48 h incubation with or without 10 ng/ml TGF- β , and tested by ELISA for presence of PDGF-AB (ELISA kit from R&D Systems, Minneapolis, MN). PDGF is expressed as pg/10⁵ cells in culture, and the bars represent the mean and SD from replicate cultures.

Figure 4

A. PDGFR β phosphorylation in hFOB1.19 cells

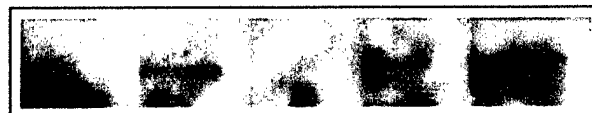
IP: anti-PDGFR β Blot: anti-phosphotyrosine



PDGF (ng/ml) 0 0.1 1.0 5.0 10 25

B. Inhibition of phosphorylation with tyrosine kinase inhibitor STI571

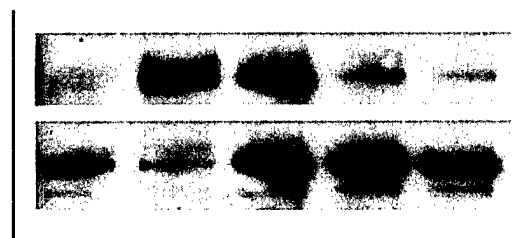
IP: anti-PDGFR β Blot: anti-phosphotyrosine



PDGF (5ng/ml) - + + + +
STI571 (uM) - - 1.0 0.5 0.1

C. PDGFR-activation in hFOB1.19 cells exposed to breast cancer conditioned medium

IP: anti-PDGFR β Blot: anti-phosphotyrosine



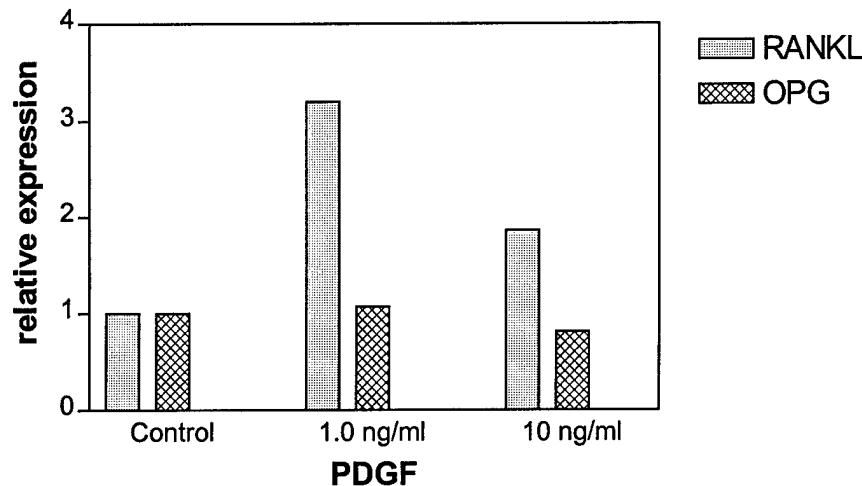
PDGF (5ng/ml) - + + - -
STI571 (0.5uM) - - + - +
BBM CM - - - + +

phospho-PDGFR β

total PDGFR β

Figure 5:

Expression of RANKL and OPG in hFOB1.19 osteoblasts stimulated with PDGF



Total RNA was isolated from hFOB1.19 cells incubated for 24 h with PDGF (1 or 10 ng/ml) and relative levels of RNA for RANKL and OPG measured by quantitative PCR. Total RNA was reversed transcribed with random primers from the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City,CA). The Gene AMP PCR system 9700 thermal cycler was used to perform the RT step using the following cycle conditions: 25°C for 10 min, 37° C for 120 min. cDNA was amplified in duplicate samples using the ABI 7000 Sequence Detection System for the expression of RANKL, OPG and 18S using TaqMan® Assay Reagents (Applied Biosystems) and following the manufacturer's recommended amplification procedure. Results were recorded as mean Ct, and relative expression was determined using the comparative Ct method. The ΔCt was calculated as the difference between the average Ct value of the endogenous control, 18s, from the average Ct value of the cytokine of interest. To compare the relative amount of target gene expression in different samples, human placenta RNA (Promega, Madison, WI) was used as a calibrator. The $\Delta\Delta Ct$ was determined by subtracting the ΔCt of the calibrator from the ΔCt of the test sample. Relative expression of of the target gene is calculated by the formula, $2^{-\Delta\Delta Ct}$, which is the amount of gene product, normalized to the endogenous control and relative to a calibrator.